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(54) Title: PROCESS FOR PRODUCING CHEESE

(57) Abstract: The present invention relates to a process for producing cheese from enzyme-treated cheese milk, and the use of the resulting produced cheese as ingredient in food products. More particularly, the present invention relates to a process for producing cheese from cheese milk treated with phospholipase A and lysophospholipase.

PROCESS FOR PRODUCING CHEESE

TECHNICAL FIELD

The present invention relates to a process for producing cheese from enzyme-treated cheese milk, and the use of the resulting produced cheese as ingredient in food products.

BACKGROUND OF THE INVENTION

In cheese products, the state of the fat phase is important to the properties of the cheese. The fat phase is particularly important for the stabilisation of the cheese during production and ripening, but also for the final cheese to be used, eaten as such, or used in prepared ready-to-eat dishes e.g. pizza, toast or burgers.

Also, the oiling-off properties of cheese products are important quality parameters. Oiling-off is the tendency to form free oil upon storage and melting. Excessive oiling-off is a defect most often related to heated products wherein cheese is used, e.g. pizza and related foods (cf. e.g. Kindstedt J.S; Rippe J.K. 1990, J Dairy Sci. 73: 867-873. It becomes more and more important to control/eliminate this defect, as the consumer concern about dietary fat levels increases. Free oil/fat in a product is perceived as a high fat content, and is generally undesirable.

There is a need for an improved process for the manufacturing of cheese, in particular a process for improving the stability of the fat in cheese.

WO 00/54601 discloses a method for improving the properties of cheese, particularly the stability of the fat phase, comprising the steps of a) treating the cheese milk with a phospholipase and b) producing cheese from the cheese milk. The phospholipase is selected from the group consisting of phospholipase A_1, phospholipase A_2, phospholipase B and combinations thereof and added in an amount sufficient to decrease the oiling-off effect in cheese and/or to increase cheese yield.

SUMMARY OF THE INVENTION

The present invention relates to a process for producing cheese comprising adding to the cheese milk a combination of phospholipase A and a lysophospholipase; in an amount effective to decrease the oiling-off effect in cheese and/or to increase cheese yield, and producing cheese from the cheese milk.

The invention further relates to a process for producing cheese comprising adding to the cheese milk a phospholipase selected from the group consisting of phospholipase A_1 and phospholipase A_2 and combinations thereof; and a lysophospholipase; to decrease the oiling-off effect in cheese and/or to increase cheese yield; and producing cheese from the cheese milk.

There is provided a process for improving the properties of cheeses; in particular, the fat stability of cheese and cheese milk is improved by the present invention. Although not limited to any theory of operation, it is believed that the addition of a phospholipase A and a lysophospholipase to the cheese milk during cheese making enhances the stability during a heat treatment of cheese produced from said treated cheese milk. By the process of the invention is also provided a method for increasing the yield in cheese production.

The invention further relates to the combined use of phospholipase A and lysophospholipase in the manufacturing of cheese products, wherein the phospholipase A and lysophospholipase treatment is conducted on the cheese milk during the production of the cheese. The invention also relates to cheeses obtainable, in particular obtained, by any of the processes described herein.

The invention also relates to a process for producing cheese comprising adding to the cheese milk a purified phospholipase selected from the group consisting of phospholipase A_1 and phospholipase A_2 and combinations thereof; and a lysophospholipase; to decrease the oiling-off effect in cheese and/or to increase cheese yield; and producing cheese from the cheese milk.

DETAILED DISCLOSURE OF THE INVENTION

A process for producing cheese comprising adding to the cheese milk a combination of a phospholipase A and a lysophospholipase to decrease the oiling-off effect in cheese and/or to increase cheese yield, and producing cheese from the cheese milk. The phospholipase A is preferably selected from the group consisting of phospholipase A_1, phospholipase A_2 and combinations thereof.

The phospholipase A and lysophospholipase are added to the cheese milk during cheese making process. As used herein, the term "cheese milk" is the milk-based composition from which the cheese is prepared.

The phospholipase A and lysophospholipase may be added at any appropriate stage during the cheese making process. In a preferred embodiment of the invention, the phospholipase A and lysophospholipase are added during standardization of the fat and/or protein content of the cheese milk, more preferably, at the same time as the cheese milk is filled into the cheese vat, at the same time as the starter culture is added to the cheese milk, and at the same time as cheese rennet is added to the cheese milk.
The phospholipase A and the lysophospholipase may be added simultaneously or sequentially during the cheese making process. In one embodiment, the phospholipase A and lysophospholipase are added to the cheese milk at different stages in the process, such as, for example, the phospholipase A is added before the lysophospholipase or the lysophospholipase is added before the phospholipase A. In a preferred embodiment, the phospholipase A and lysophospholipase are added simultaneously in the cheese making process.

<u>Cheese milk and the production of cheese</u>:
In the present context, the term "cheese" refers to any kind of cheese and such as, e.g., natural cheese, cheese analogues and processed cheese. The cheese may be obtained by any suitable process known in the art, such as, e.g., by enzymatic coagulation of the cheese milk with rennet, or by acidic coagulation of the cheese milk with food grade acid or acid produced by lactic acid bacteria growth. In one embodiment, the cheese manufactured by the process of the invention is rennet-curd cheese. Rennet is commercially available, e.g. as Naturen® (animal rennet), Chy-max® (fermentation produced chymosin), Microlant®

(Microbial coagulant produced by fermentation), all from Chr. Hansen A/S, Denmark). The cheese milk may be subjected to a conventional cheese-making process.

Processed cheese is preferably manufactured from natural cheese or cheese analogues by cooking and emulsifying the cheese, such as, with emulsifying salts (e.g. phosphates and citrate). The process may further include the addition of spices/condiments.
The term "cheese analogues" refers to cheese-like products which contain fat (such as, e.g., milk fat (e.g., cream) as a part of the composition, and, in which further contain, as part of the composition, a non-milk constituents, such as, e.g., vegetable oil. An example of a cheese analogue is cheese base.

The cheeses produced by the process of the present invention comprise all varieties of cheese, such as, e.g. Campesino, Chester, Danbo, Drabant, Herregård, Manchego, Provolone, Saint Paulin, Soft cheese, Svecia, Taleggio, White cheese, including rennet-curd cheese produced by rennet-coagulation of the cheese curd; ripened cheeses such as Cheddar, Colby, Edam, Muenster, Gryere, Emmenthal, Camembert, Parmesan and Romano; fresh cheeses such as Mozzarella and Feta; acid coagulated cheeses such as cream cheese, Neufchatel, Quarg, Cottage Cheese and Queso Blanco; and pasta filata cheese. One embodiment relates to the production of pizza cheese by the process of the invention.

In cheese manufacturing, the coagulation of the casein in milk is preferably performed in one of two ways: the so-called rennet-curd and acid-curd cheese. In cheese production these two types of curds makes up two major groups of cheese types. Fresh acid-curd cheeses refer to those varieties of cheese produced by the coagulation of milk, cream or whey via acidification or a combination of acid and heat, and which are ready for consumption once the manufacturing without ripening are completed. Fresh acid-curd cheeses generally differ from rennet-curd cheese varieties (e.g. Camembert, Cheddar, Emmenthal) where coagulation normally is induced by the action of rennet at pH values 6.4-6.6, in that coagulation normally occurs close to the isoelectric point of casein, i.e. e.g. at pH 4.6 or at higher values when elevated temperatures are used, e.g. in Ricotta pH 6.0 and 80°C. In a preferred embodiment of the invention, the cheese belongs to the class of rennet curd cheeses.

Mozzarella is a member of the so-called pasta filata, or stretched curd, cheeses which are normally distinguished by a unique plasticizing and kneading treatment of the fresh curd in hot water, which imparts the finished cheese its characteristic fibrous structure and melting and stretching properties, cf. e.g. "Mozzarella and Pizza cheese" by Paul S. Kindstedt, Cheese: Chemistry, physics and microbiology, Volume 2: Major Cheese groups, second edition, page 337-341, Chapman & Hall. Pizza cheese as used herein includes cheeses suitable for pizzas and they are usually pasta filata/stretched curd cheeses. In one embodiment, the process of the invention further comprises a heat/stretching treatment as for pasta filata cheeses, such as for the manufacturing of Mozzarella.

In further embodiments of the invention, the cheese milk is prepared, totally or in part, from dried milk fractions, such as, e.g., whole milk powder, skim milk powder, casein, caseinate, total milk protein or buttermilk powder, or any combination thereof.

In preferred embodiments, the cheese milk, to which phospholipase A and lysophospholipase are to be added, comprises or consists of cream. In further embodiments, the cheese milk, to which phospholipase A and lysophospholipase are to be added, comprises or consists of butter. In still further embodiments, the cheese milk, to which phospholipase A and lysophospholipase are to be added, comprises or consists of buttermilk.

Milk from different species of animals may be used in the production of cheese. Thus, "milk" may be the lacteal secretion obtained by milking, e.g., cows, sheep, goats, buffaloes or camels.

The milk for production of cheese may be standardised to the desired composition by removal of all or a portion of any of the raw milk components and/or by adding thereto additional amounts of such components. This may be done by separation of the raw milk into cream and skim milk at arrival to the dairy. Thus, the cheese milk may be prepared as done conventionally by fractioning the raw milk and recombining the fractions so as to obtain the desired final composition of the cheese milk. The separation may be made in continuous centrifuges leading to a skim milk fraction with very low fat content (i.e. e.g. < 0.5%) and cream with e.g. > 35% fat. The cheese milk may be prepared by mixing cream and skim milk.

The cheese milk, to which phospholipase A and lysophospholipase are to be added, comprises phospholipids, such as e.g. lecithin. The cheese milk may have any total fat content which is found suitable for the cheese to be produced by the process of the invention, such as, e.g., about 25% fat (of dry matter), such as e.g. in the range 10-50% fat, of which, e.g., about 0.06% is phospholipids, such as e.g. 0.02-5% (w/w) of the total fat content is phospholipids.

Conventional steps may be taken to secure low bacterial counts in the cheese milk. It is generally preferred not to pasteurise the skim milk because heat denatured proteins in the cheese milk have a negative influence on the coagulation of the milk, and retard the ripening of the cheese. The bacterial count of the skim milk fraction may thus be lowered by other technologies, such as, for example, by microfiltration or bactofugation. The cream is preferably pasteurised to lower the bacterial count in the product. In another preferred embodiment, the cheese milk is raw, unpasteurised milk.

In an embodiment of the invention, the cheese milk may be subjected to a homogenization process before the production of cheese, such as e.g. in the production of Danish Blue Cheese.

The enzymatic treatment:

The enzymatic treatment in the process of the invention may be conducted by dispersing the phospholipase A and lysophospholipase into cheese milk, and allowing the enzyme reaction to take place at an appropriate holding-time at an appropriate temperature. The treatment with phospholipase A and lysophospholipase may be carried out at conditions chosen to suit the selected enzymes according to principles well known in the art.

The enzymatic treatment may be conducted at any suitable pH, such as e.g., in the range 2-10, such as, at a pH of 4-9 or 5-7. It may be preferred to let the phospholipase A and lysophospholipase act at the natural pH of the cheese milk as it develops during the cheese making process.

The process may be conducted so that the phospholipase A and lysophospholipase are allowed to react at coagulation temperature, such as, 25-45°C (e.g., for at least 5 minutes, such as, e.g., for at least 10 minutes or at least 30 minutes, e.g., for 5-60 minutes).

Optionally, after the phospholipase A and the lysophospholipase have been allowed to act on the cheese milk, the phospholipase A and/or lysophospholipase enzyme protein is removed, reduced, inactivated or any combination thereof.

The phospholipase A and the lysophospholipase are added in suitable amounts to produce the cheese having the desired properties. Preferably, the phospholipase A and lysophospholipase are added in amounts effective to decrease the oiling-off effect in cheese and/or to increase cheese yield. A suitable dosage of phospholipase A will usually be in the range 0.003-0.3 mg enzyme protein per g milk fat, preferably 0.01-0.3 mg enzyme protein per g milk fat, more preferably, 0.03 mg enzyme protein per g milk fat.
Dosage of lysophospholipase will usually be in the range 0.005-0.5 mg enzyme protein per g milk fat, preferably 0.01-0.5 mg enzyme protein per g milk fat, more preferably 0.05 mg enzyme protein per g milk fat.

<u>Enzymes to be used in the process of the invention:</u>

Phospholipids, such as lecithin or phosphatidylcholine, consist of glycerol esterified with two fatty acids in an outer (sn-1) and the middle (sn-2) positions and esterified with phosphoric acid in the third position; the phosphoric acid, in turn, may be esterified to an amino-alcohol. Phospholipases are enzymes which participate in the hydrolysis of phospholipids. Several types of phospholipase activity can be distinguished, including phospholipases A_1 and A_2 which hydrolyze one fatty acyl group (in the sn-1 and sn-2 position, respectively) to form lysophospholipid. Lysophospholipase hydrolyzes the remaining fatty acyl group in lysophospholipid.

The enzymes used in the process of the present invention comprise a phospholipase A and a lysophospholipase. The term phospholipase A includes phospholipase A_1, phospholipase A_2 and the combination of phospholipase A_1 and phospholipase A_2, such as e.g. the combination of an enzyme with phospholipase A_1 activity and an enzyme with phospholipase A_2 activity, or a single enzyme with both phospholipase A_1 and phospholipase A_2 activity.

<u>Phospholipase A_1</u> is defined according to standard enzyme EC-classification as EC 3.1.1.32.

Official Name: Phospholipase A_1.

Reaction catalyzed:

phosphatidylcholine + H(2)O <>

2-acylglycerophosphocholine + a fatty acid anion
Comment(s):
has a much broader specificity than EC 3.1.1.4.

Phospholipase A_2 is defined according to standard enzyme EC-classification as EC 3.1.1.4
Official Name: phospholipase A_2.
Alternative Name(s):phosphatidylcholine 2-acylhydrolase.
lecithinase a; phosphatidase; or phosphatidolipase.
Reaction catalysed:
phosphatidylcholine + h(2)o <>
1-acylglycerophosphocholine + a fatty acid anion
comment(s): also acts on phosphatidylethanolamine, choline plasmalogen and phosphatides, removing the fatty acid attached to the 2-position.

Lysophospholipase is defined according to standard enzyme EC-classification as EC 3.1.1.5.
Official Name:lysophospholipase.
Alternative Name(s):lecithinase b; lysolecithinase;
phospholipase B; or PLB.
Reaction catalysed:
2-lysophosphatidylcholine + h(2)o <> glycerophosphocholine + a fatty acid anion.

The phospholipase A activity may be provided by enzymes having other activities as well, such as e.g. a lipase with phospholipase A activity. The phospholipase A activity may e.g. be from a lipase with phospholipase A side activity. In other embodiments of the invention the phospholipase A enzyme activity is provided by an enzyme having essentially only phospholipase A activity and wherein the phospholipase A enzyme activity is not a side activity.

The phospholipase A may be of any origin, e.g. of animal origin (such as, e.g. mammalian), e.g. from pancreas (e.g. bovine or porcine pancreas), or snake venom or bee venom. Alternatively, the phospholipase A may be of microbial origin, e.g. from filamentous fungi, yeast or bacteria, such as the genus or species *Aspergillus*, e.g. *A. niger*; *Dictyostelium*, e.g. *D. discoideum*; *Mucor*, e.g. *M. javanicus*, *M. mucedo*, *M. subtilissimus*; *Neurospora*, e.g. *N. crassa*; *Rhizomucor*, e.g. *R. pusillus*; *Rhizopus*, e.g. *R. arrhizus*, *R. japonicus*, *R. stolonifer*; *Sclerotinia*, e.g. *S. libertiana*; *Trichophyton*, e.g. *T. rubrum*; *Whetzelinia*, e.g. *W.*

sclerotiorum; *Bacillus*, e.g. *B. megaterium*, *B. subtilis*; *Citrobacter*, e.g. *C. freundii*; *Enterobacter*, e.g. *E. aerogenes*, *E. cloacae Edwardsiella*, *E. tarda*; *Erwinia*, e.g. *E. herbicola*; *Escherichia*, e.g. *E. coli*; *Klebsiella*, e.g. *K. pneumoniae*; *Proteus*, e.g. *P. vulgaris*; *Providencia*, e.g. *P. stuartii*; *Salmonella*, e.g. *S. typhimurium*; *Serratia*, e.g. *S. liquefasciens*, *S. marcescens*; *Shigella*, e.g. *S. flexneri*; *Streptomyces*, e.g. *S. violeceoruber*, *Yersinia*, e.g. *Y. enterocolitica*. Thus, the phospholipase A may be fungal, e.g. from the class *Pyrenomycetes*, such as the genus *Fusarium*, such as a strain of *F. culmorum*, *F. heterosporum*, *F. solani*, or a strain of *F. oxysporum*. The phospholipase A may also be from a filamentous fungus strain within the genus *Aspergillus*, such as a strain of *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus niger* or *Aspergillus oryzae*. A preferred phospholipase A is derived from a strain of *Fusarium*, particularly *F. oxysporum*, e.g. from strain DSM 2672 as described in WO 98/26057, especially described in claim 36 and SEQ ID NO. 2 of WO 98/26057. In further embodiments, the phospholipase A is a phospholipase as disclosed in WO 00/32758 (Novozymes A/S, Denmark).

Lysophospholipase

The term "lysophospholipase" used herein in connection with an enzyme of the invention is intended to cover an enzyme with lysophospholipase activity.

The lysophospholipase activity may be provided by enzymes having other activities as well, such as e.g. a lipase with lysophospholipase activity. The lysophospholipase activity may e.g. be from a lipase with lysophospholipase side activity. In other embodiments of the invention the lysophospholipase enzyme activity is provided by an enzyme having essentially only lysophospholipase activity and wherein the lysophospholipase enzyme activity is not a side activity. In one embodiment of the invention, the lysophospholipase is not lipases having lysophospholipase side activity as defined in WO 98/26057.

The lysophospholipase may be of any origin, e.g. of animal origin (such as, e.g. mammalian), e.g. from liver (e.g. rat liver). Alternatively, the lysophospholipase may be of microbial origin, e.g. from filamentous fungi, yeasts or bacteria, such as the genus or species *Aspergillus*, e.g. *A. foetidus*, *A. fumigatus*, *A. nidulans*, *A. niger*, *A. oryzae*; *Botrytis*, e.g. *B. cinerea*; *Candida*, e.g. *C. albicans*; *Cryptococcus*, e.g. *C. neoformans*, *Escherichia*, e.g. *E. coli*, *Fusarium*, e.g. *F. sporotrichioides*, *F. venenatum*, *F. verticillioides*; *Hyphozyma*; *Kluyveromyces*, e.g. *K. lactis*; *Magnaporte*, e.g. *M. grisea*; *Metarhizium*, e.g. *M. anisopliae*; *Mycosphaerella*, e.g. *M. graminicola*; *Neurospora*, e.g. *N. crassa*; *Penicillium*, e.g. *P.*

notatum; Saccharomyces, e.g. *S. cerevisiae; Schizosaccharomyces*, e.g. *S. pombe; Torulaspora*, e.g. *T. delbrueckii; Vibrio*; e.g. *V. cholerae*. A preferred lysophospholipase is derived from a strain of *Aspergillus*, particularly lysophospholipase LLPL-1 or LLPL-2 from *A. niger*, e.g. as contained in the *Escherichia coli* clones DSM 13003 or DSM 13004, or lysophospholipase LLPL-1 or LLPL-2 from *A. oryzae*, e.g. as contained in the *E. coli* clones DSM 13082 or DSM 13083 as described in WO 01/27251, especially described in claim 1 and SEQ ID NOs. 2, 4, 6 or 8 of WO 01/27251.

<u>Enzymes with both phospholipase A and lysophospholipase activity</u>

In a preferred embodiment of the invention the phospholipase A and the lysophospholipase activities are provided by a single enzyme having both phospholipase A activity and lysophospholipase activity. E.g. in one embodiment a single enzyme has both phospholipase A_1 activity and lysophospholipase activity, in another embodiment a single enzyme has both phospholipase A_2 activity and lysophospholipase activity, and in a further embodiment a single enzyme has both phospholipase A_1 activity, phospholipase A_2 activity, and lysophospholipase activity.

The invention thus also relates to a process for producing cheese comprising adding to the cheese milk an enzyme with both phospholipase A activity and lysophospholipase activity to decrease the oiling-off effect in cheese and/or to increase cheese yield; and producing cheese from the cheese milk.

Enzymes with both phospholipase A and lysophospholipase activity are known to the person skilled in the art. Enzymes with both phospholipase A and lysophospholipase activity are e.g. described by Saito et al., Methods in Enzymology (1991) 197, 446-456, and by Lee et al., J. Biol. Chem. (1994) 269, 19725-19730.

<u>Enzyme sources and formulation</u>

The phospholipase A and/or lysophospholipase used in the process of the invention may be derived or obtainable from any of the sources mentioned herein. The term "derived" means in this context that the enzyme may have been isolated from an organism where it is present natively, i.e. the identity of the amino acid sequence of the enzyme are identical to a native enzyme. The term "derived" also means that the enzymes may have been produced recombinantly in a host organism, the recombinant produced enzyme having either an identity identical to a native enzyme or having a modified amino acid sequence, e.g. having

one or more amino acids which are deleted, inserted and/or substituted, i.e. a recombinantly produced enzyme which is a mutant and/or a fragment of a native amino acid sequence. Within the meaning of a native enzyme are included natural variants. Furthermore, the term "derived" includes enzymes produced synthetically by e.g. peptide synthesis. The term "derived" also encompasses enzymes which have been modified e.g. by glycosylation, phosphorylation etc., whether in vivo or in vitro. The term "obtainable" in this context means that the enzyme has an amino acid sequence identical to a native enzyme. The term encompasses an enzyme that has been isolated from an organism where it is present natively, or one in which it has been expressed recombinantly in the same type of organism or another, or enzymes produced synthetically by e.g. peptide synthesis. With respect to recombinantly produced enzyme the terms "obtainable" and "derived" refer to the identity of the enzyme and not the identity of the host organism in which it is produced recombinantly.

Accordingly, the phospholipase A and/or lysophospholipase may be obtained from a microorganism by use of any suitable technique. For instance, a phospholipase A and/or lysophospholipase enzyme preparation may be obtained by fermentation of a suitable microorganism and subsequent isolation of a phospholipase A and/or lysophospholipase preparation from the resulting fermented broth or microorganism by methods known in the art. The phospholipase A and/or lysophospholipase may also be obtained by use of recombinant DNA techniques. Such method normally comprises cultivation of a host cell transformed with a recombinant DNA vector comprising a DNA sequence encoding the phospholipase A or lysophospholipase in question and the DNA sequence being operationally linked with an appropriate expression signal so that it is capable of expressing the phospholipase A or lysophospholipase in a culture medium under conditions permitting the expression of the enzyme and recovering the enzyme from the culture. The DNA sequence may also be incorporated into the genome of the host cell. The DNA sequence may be of genomic, cDNA or synthetic origin or any combinations of these, and may be isolated or synthesized in accordance with methods known in the art.

Suitable phospholipases are available commercially. As typical examples of the enzymes for practical use, pancreas-derived phospholipase A_2 such as Lecitase® (manufactured by Novozymes A/S) is preferably used. A suitable lysophospholipase is e.g. *Aspergillus niger* lysophospholipase LLPL-2 that can be produced recombinantly in *A. niger* as described in WO 01/27251.

In the process of the invention the phospholipase A and/or lysophospholipase may be purified. The term "purified" as used herein covers phospholipase A or lysophospholipase enzyme protein free from components from the organism from which it is derived. The term "purified" also covers phospholipase A or lysophospholipase enzyme protein free from components from the native organism from which it is obtained, this is also termed "*essentially* pure" phospholipase A or lysophospholipase and may be particularly relevant for phospholipases and lysophospholipases which are naturally occurring and which have not been modified genetically, such as by deletion, substitution or insertion of one or more amino acid residues.

Accordingly, the phospholipase A and/or lysophospholipase may be purified, viz. only minor amounts of other proteins being present. The expression "other proteins" relate in particular to other enzymes. The term "purified" as used herein also refers to removal of other components, particularly other proteins and most particularly other enzymes present in the cell of origin of the phospholipase A or lysophospholipase. The phospholipase A or lysophospholipase may be "substantially pure", i.e. free from other components from the organism in which it is produced, i.e., e.g., a host organism for recombinantly produced phospholipase A or lysophospholipase. Preferably, the enzymes are at least 75% (w/w) pure, more preferably at least 80%, 85%, 90% or even at least 95% pure. In a still more preferred embodiment the phospholipase A or lysophospholipase is an at least 98% pure enzyme protein preparation. In other embodiments the phospholipase A and/or lysophospholipase is not naturally present in milk.

The terms "phospholipase" and "lysophospholipase" include whatever auxiliary compounds that may be necessary for the catalytic activity of the enzyme, such as, e.g. an appropriate acceptor or cofactor, which may or may not be naturally present in the reaction system.

The phospholipase A and lysophospholipase may be in any form suited for the use in question, such as e.g. in the form of a dry powder or granulate, a non-dusting granulate, a liquid, a stabilized liquid, or a protected enzyme. Granulates may be produced, e.g. as disclosed in US 4,106,991 and US 4,661,452, and may optionally be coated by methods known in the art. Liquid enzyme preparations may, for instance, be stabilized by adding stabilizers such as a sugar, a sugar alcohol or another polyol, lactic acid or another organic acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

By the process of the invention, the lecithin content of the cheese may be reduced by at least 5%, such as at least 10%, at least 20%, at least 30%, at least 50%, such as in the range of 5-95% compared to a similar cheese making process but without the enzymatic treatment of a phospholipase A and a lysophospholipase, as described herein.

In cow milk, the lecithin constitutes normally more than 95% of the phospholipids in milk whereas the lysolecithin is approximately 1% of the phospholipids. Although the phospholipids represent normally less than 1% of the total lipids in cow milk, they play a particularly important role, being present mainly in the milk fat globule membrane. By the process of the present invention the lecithin content in the obtainable cheese may be less than 90%, such as e.g. less than 80%, e.g. less than 60% or less than 50% of the total content of phospholipid in the cheese. The lecithin content may be measured by any method known by the skilled person, e.g. by HPLC.

The present invention further relates to use of the cheese produced by the process of the invention in pizza, ready-to-eat dishes, processed cheese or as an ingredient in other food products. Accordingly, the cheese produced according to the process of the invention may be used in further processed food products like processed cheese, pizza, burgers, toast, sauces, dressings, cheese powder or cheese flavours.

In further embodiments, the process of the invention further comprises the step of subjecting the cheese to a heating treatment, such as, e.g., in the range 150-350°C.

The invention also relates to a cheese obtainable, in particular obtained, by the process of the invention.

The present invention is further illustrated in the following example which is not to be in any way limiting to the scope of protection.

Example 1

Cheese produced with addition of phospholipase A and lysophospholipase

Enzyme preparations

A: 0.03 mg enzyme protein per g fat of phospholipase A (Lecitase® 10 L manufactured by Novozymes A/S, Denmark)

B: 0.05 mg per g milk fat of lysophospholipase (*Aspergillus niger* lysophospholipase LLPL-2 produced recombinantly in *A. niger* as described in WO 01/27251).

C: The combination of A and B above

Cheese production

Pasteurized, non-homogenized cream (North Carolina State University Dairy Plant) was used to standardize pasteurized, non-homogenized skim milk (North Carolina State University Dairy Plant) to 3.5% fat for production of full fat Mozzarella cheese.

Starter culture was prepared by adding 0.1 g Rhodia LH100 and 0.3 g Rhodia TA061 starter cultures to 50 ml of the skim milk and equilibrating to 35°C with gentle, continuous stirring. Standardised cheese milk was equilibrated to 35°C and divided into 3 batches. 1.4 % starter culture was added to each batch, and at the same time the enzyme preparations A, B and C above were added to the respective batches under gentle stirring.

When pH reached 6.45 – 6.50, 0.01 % rennet (Chymax, fermentation produced chymosin, manufactured by Chr. Hansens Lab., Denmark) was added to each batch under vigorous stirring for three minutes. The stirrers were removed and the milk was allowed to coagulate. The appropriate cutting time was determined by making a downward cut into the curd with knife or spatula. The spatula was then inserted into the curd below the cut and perpendicular to the direction of the cut and used to raise the curd upward. The curd was judged ready for cutting when the curd separated upon lifting and sharp edges were maintained on the top surface at the edge of the cut. Cutting was performed with ½ inch knives and the curd was left to rest for 5 minutes after cutting.

The temperature was increased to 41°C under gentle and intermittently stirring and kept until pH reached 5.65 – 5.70. Then the whey was drained off and curd particles poured into stainless steel bowls. The bowls were floated in 41°C water bath to maintain curd

temperature and excess whey was drained off periodically, leaving only enough to cover curds for maintenance of heat.

When curd pH reached 5.25 - 5.3, all whey was drained off and the curd flooded with deionised water at 57°C for 5 min. The curd was stretched by hand for 1 min and returned to 57°C water to restore curd temperature when necessary. The stretched curd was immersed in room temperature tap water for 10 – 15 minutes to cool, then blotted dry, and refrigerated.

Determination of Oiling-Off:

Cheese was ground in a blender for 20 seconds for uniformity of sample. About 3.0 grams were moulded into a metal ring (2.2 cm diameter), placed in the centre of a Whatman #4 filter paper and placed into an oven set at 90°C for 5 minutes to melt. Each sample was tested in triplicate. Oiling-off was determined by image analysis, as the difference in the areas between the ring of oil developed during melting around the cheese, and the circle of cheese. The calculation for oiling off was as follows:

$$\text{Oiling-Off} = \frac{\text{Total Area - Area of Cheese}}{\text{Area of Cheese}} \times 100$$

Results

The results (protein, moisture, fat and oiling off) are shown in table 1 below.

Table 1

	A Phospholipase A	B Lysophospholipase	C Phospholipase A and Lysophospholipase
Protein (%)	26.1	24.8	26.6
Moisture (%)	50.9	51.2	49.7
Fat (%)	22	22	23
Oiling off (ratio)	84	105	68

From the results in table 1, it can be seen that the addition of the phospholipase A has a higher effect on reducing oiling off in the cheese than addition of the lysophospholipase.

When both enzymes are added, however, oiling off is even lower, showing the synergistic effect of the two enzymes.

Example 2

Formation of long chain fatty acids in milk reacted with phospholipase A and lysophospholipase

Substrate Preparation and Enzyme Treatment. Cream was standardized to a fat content of 25% using skim milk. Milk samples were treated with phospholipase A (Lecitase® 10 L manufactured by Novozymes A/S, Denmark), Lysophospholipase (*Aspergillus niger* lysophospholipase LLPL2 expressed recombinantly in *A. niger* as described in WO 01/27251) and combinations of phospholipase A and lysophospholipase as indicated in table 3. The enzymes were pre-diluted in water, if necessary, and dosed into the cream at the appropriate concentration. Samples were incubated at 35°C for 1.5 hr without shaking. Reactions were stopped by the addition of organic solvent for lipid extraction.

Lipid Extraction. Total milk lipids were extracted by mixing each sample with 1 ml of water followed by 9 ml of chloroform/methanol (2:1). Samples were mixed vigorously for 1 min and centrifuged at 3000 rpm for 5 min. Six milliliters of the lower $CHCl_3$ phase were removed and dried down under vacuum. Samples were reconstituted in 2 ml of $CHCl_3$. Fatty Acids were further purified by solid phase extraction (SPE). Each $CHCL_3$ extract was applied to an aminopropyl SPE column under vacuum. The column was washed with 4 ml of $CHCl_3$/isopropanol (2:1) to remove neutral lipids. Fatty Acids were eluted with 4 ml of diethylether acidified with glacial acetic acid (2% v/v). Ether extracts were dried down in a rotary evaporator and reconstituted in 1.0 ml of methanol for HPLC analysis.

HPLC Method: Reverse phase chromatography was conducted using a Luna C8 (150 x 4.6 mm, 5 μ, 100 A) column from Phenomenex (Torrance, CA USA), and a mobile phase of acetonitrile and water containing 0.1% trifluoroacetic acid. See Table 2 for the gradient elution scheme. Detection of eluted Fatty Acids was done by evaporative light scattering. HPLC Fatty Acid standards (linoleic, palmitic, oleic, and stearic acids) were chosen based on their prevalence in total lipids of bovine milk. Stock solutions were routinely prepared in 100% methanol in the concentration range of 2-10 mg/ml. HPLC calibrators were prepared

from Fatty Acid stock solutions by dilution to the appropriate concentration in 100% methanol.

Table 2. Gradient Elution Profile for HPLC Fatty Acid Analysis.

Step No.	Step Time (minutes)	%A	%B
1	0	80	20
2	2	80	20
3	12	90	10
4	17	90	10
5	19	80	10

A = 100% Acetonitrile

B = Water/0.1% Trifluoroacetic acid

The amount of generated free long chain fatty acids after treatment of the cream samples with phospholipase A and/or lysophospholipase is shown in table 3. It can be seen that increasing the amount of phospholipase A from 0.04 mg/(g fat) to 0.18 mg/(g fat) does not increase the amount of generated free fatty acids considerably. When lysophospholipase is added in combination with the phospholipase A, however, the amount of generated fatty acid is considerably increased.

Table 3. Amount of generated free long chain fatty acid after treatment of cream with phospholipase A and/or lysophospholipase

Sample	Lecitase® 10 L (mg enzyme protein/g milk fat)	LLPL2 (mg enzyme protein/g milk fat)	Generated Fatty acid (mg/g milk fat)
1	0.04	0	1.05
2	0.18	0	1.12
3	0.04	0.12	1.73
4	0.18	0.12	1.74
5	0.18	0.24	1.80
6	0.18	0.48	1.87
7	0.18	0.96	1.86

Claims

1. A process for producing cheese comprising adding to the cheese milk a phospholipase selected from the group consisting of phospholipase A_1 and phospholipase A_2 and combinations thereof; and a lysophospholipase; to decrease the oiling-off effect in cheese and/or to increase cheese yield; and producing cheese from the cheese milk.

2. The process of claim 1, wherein the amount of phospholipase A is in the range 0.003-0.3 mg enzyme protein per g milk fat

3. The process of claim 1, wherein the amount of phospholipase A is in the range 0.01-0.3 mg enzyme protein per g milk fat

4. The process of claim 1, wherein the amount of phospholipase A is 0.03 mg enzyme protein per g milk fat

5. The process of any of the claims 1-4, wherein the phospholipase A and/or lysophospholipase is added to the cheese milk during standardization of the fat and/or protein content of the cheese milk

6. The process of any of the claims 1-4, wherein the phospholipase A and/or lysophospholipase is added at the same time as the starter culture.

7. The process of any of the claims 1-4, wherein the phospholipase A and/or lysophospholipase is added at the same time as the cheese rennet.

8. The process according to any of the claims 1-7, further comprising the step of removing or reducing the content of the phospholipase A and/or lysophospholipase enzyme protein after the enzymes have been allowed to act in the cheese milk.

9. The process according to any of the claims 1-8, further comprising the step of inactivating the phospholipase A and/or lysophospholipase after the enzymes have been allowed to act in the cheese milk.

10. The process according to any of the claims 1-9, wherein the cheese is selected from the group consisting of rennet-curd cheese produced by rennet-coagulation of the cheese curd; ripened cheeses, fresh cheeses, and acid coagulated cheeses.

11. The process according to any of the claims 1-9, wherein the cheese is selected from the group consisting of Campesino, Chester, Danbo, Drabant, Herregård, Manchego, Provolone, Saint Paulin, Soft cheese, Svecia, Taleggio, White cheese, Cheddar, Colby, Edam, Muenster, Gryere, Emmenthal, Camembert, Parmesan, Romano, Mozzarella, Feta; cream cheese, Neufchatel, Quarg, and Queso Blanco.

12. The process according to any of the claims 1-10, wherein the cheese milk is subjected to a homogenization step before the production of cheese.

13. The process of claim 12, wherein the cheese is Danish Blue Cheese.

14. The process of any of claims 1-13 wherein the phospholipase A and the lysophospholipase activities are provided by a single enzyme having both phospholipase A activity and lysophospholipase activity.

15. The process according to any of the claims 1-14, further comprising the step of processing the cheese into a food product.

16. The process according to claim 15, wherein said food product is selected from the group consisting of pizza, ready-to-eat dishes, toast, burgers, lasagna, dressing, sauces, cheese powder, cheese flavor, and processed cheese.

17. A cheese prepared by the process of any of the claims 1-14.

INTERN[redacted]NAL SEARCH REPORT

PCT/[redacted]3/00113

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A23C19/032 A23C19/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	page 10, line 19 - line 20; claims 1-38 page 17, line 24 -page 18, line 10 page 9, line 28 - line 30	14

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
4 June 2003	30/06/2003

Name and mailing address of the ISA	Authorized officer
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